

THE EFFECT OF DRY GRINDING ON THE PROPERTIES OF PROTEINS

BY
HAROLD ROBERT COHEN

B. S., University of Chicago, 1938
M. S., University of Illinois, 1939

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHYSIOLOGICAL CHEMISTRY
IN THE GRADUATE SCHOOL OF THE
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The Effect of Dry Grinding on the Properties of Proteins 1. Native, Denatured and Coagulated Ovalbumin*

Harold R. Cohen

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Received November 27, 1942

Physical agents such as shaking, high pressure, formation of surface films and supersonic waves are capable of bringing about denaturation and coagulation of proteins. The number of different proteins studied up to this time have been too few to warrant including grinding as another important physical agent bringing about changes in the protein molecule which are associated with denaturation and coagulation. In this and subsequent papers it will be shown that the dry grinding of numerous proteins produces definite irreversible changes in the protein molecule.

Our attention was brought to this problem when it was found that collagen yielded some water soluble protein after having been ground in the ball mill.

Several investigators have shown that dry grinding has a definite effect on the proteins and polysaccharides they studied.

Kuhne (1) found that pepsin was able to digest hair keratin after it had been subjected to grinding. Alsberg and Perry (2) ground starch and found that it became soluble in cold water. Alsberg and Griffith (3) observed that gelatin became soluble in cold water after being ground in the ball mill. These authors ground wheat flour and found that the water insoluble colloids, gliadin and glutenin, were not rendered materially soluble by 16 hours grinding. Gluten prepared from excessively ground flour swells less than the control gluten. They concluded that grinding modifies the swelling property of gels by modifying the protein structure.

Boissevain (4) repeated the investigations of Alsberg and coworkers

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and found that the effect of grinding on starch was the same as the action of other hydrolytic agents. He also ground tubercle bacilli and obtained water soluble protein, peptone and polysaccharide. This author compares the effect of grinding to that of acid hydrolysis.

Routh and Lewis (5) compared the rates of digestion of wool ground for 75 and 175 hours with that of keratin produced by the reduction of wool with thioglycolic acid (6). The ground wool contained water soluble protein which gave a positive Biuret test, a slight precipitate with trichloroacetic acid and positive Folin and Marenzi and cyanide nitroprusside reactions. 36.6 per cent of the total nitrogen and 34.1 per cent of the sulfur were lost when the ground powder was dialyzed against running water.

Routh (7) carried out chemical studies on the effect of grinding on wool. The cystine and total nitrogen in the water soluble fraction increased with the time of grinding. The amino nitrogen varied but slightly and was practically constant and independent of the amount of grinding. Routh points out that "Boissevain's results indicate that the process is similar to acid hydrolysis. . . . Hydrolytic action alone cannot explain the results obtained in the present investigation. The mechanical process may produce a water soluble fraction merely by bringing the water soluble material into contact with the solvent. Whatever the process of degradation it is accompanied by oxidative changes."

It shall be our purpose in this and succeeding papers to show that dry grinding produces changes in the protein molecule which warrant its inclusion as another physical agent producing changes associated with denaturation and coagulation.

EXPERIMENTAL

The proteins studied and reported in this investigation were all ground under the following conditions. The dried proteins were placed in porcelain ball mills of one liter or eight liters capacity which were filled to half capacity with flint pebbles, the average diameter of which was $\frac{1}{2}$ ". The ball mill was rotated at approximately 100 revolutions per minute.

Native Ovalbumin: The ovalbumin used in this experiment was prepared by the method of Cole (8) and was recrystallized 4 times. Five grams of dry, crystallized ovalbumin were placed in a ball mill of one liter capacity and ground for 24 hours. The dry powder was removed from the mill and separated from the stones by passing through a 40 mesh sieve.

The ground powder was found to be mainly insoluble in water. The water soluble portion which was small in quantity gave a positive Biuret, was precipitated by half saturation with ammonium sulfate and was not coagulated by heating at the isoelectric point. The water insoluble residue contained an acid soluble fraction, soluble at pH 3.0 (HCl) and an alkali soluble fraction soluble at pH 9.5 (NaOH). The amount of protein dissolved in both cases was small and precipitated when the solutions were adjusted to the isoelectric point. These fractions have the solubilities of acid and alkali denatured proteins. The major portion of the ground ovalbumin was insoluble in water, dilute acid and alkali, and thus has a solubility similar to that of coagulated ovalbumin.

The residue insoluble in water, acid and alkali contained some protein soluble in one-half saturated urea solution. The remaining insoluble protein formed a highly opalescent solution of high viscosity in saturated urea solution, which after standing for a short time, became a gel.

TABLE I

Chemical Differences in Native Ovalbumin and Water Soluble Fractions

	Per cent Nitrogen	Per cent Sulfur	Per cent Tyrosine	Per cent Tryptophan
Preparation I.....	15.60	1.34	4.20	1.30
Preparation II.....	15.27	2.94	2.46	0
Preparation III.....	15.13	2.20	3.06	0.51

Coagulated ovalbumin required standing in saturated urea for 3-4 days before it disperses and forms a gel, never actually dissolving.

Acid Denatured Ovalbumin: The acid denatured ovalbumin was obtained by heating a dilute salt free solution of ovalbumin at pH 3 in a water bath at 100°C. for 20 minutes. The denatured protein was precipitated by adjusting the solution to the isoelectric point, pH 4.8, filtered on a Buchner funnel and spread on plates to dry. Five grams of dry denatured ovalbumin were ground in a mill of one liter capacity for a period of 36 hours. The ground powder was now almost entirely insoluble at pH 3.0, whereas the dry unground protein was approximately 50 per cent soluble at that pH. The acid soluble ovalbumin has been converted into an acid insoluble form.

Heat Coagulated Ovalbumin: The heat coagulated ovalbumin was prepared by heating a dilute solution of ovalbumin at its isoelectric pH and the coagulum was dried by passing a current of dried warm air over it. Thirty grams of dry coagulated ovalbumin were ground in a ball mill of eight liters capacity for a period of 48 hours. The powder was

extracted with distilled water until all of the water soluble protein was removed. The water soluble fraction was dried by evaporation at 38°C. in a current of dry air and represents Preparation II.

The insoluble residue was dried and again ground for 48 hours. The powder was extracted with water, dried and designated as Preparation III. An unground sample of dried crystallized ovalbumin constitutes Preparation I.

The nitrogen of the water soluble fractions was determined by the Kjeldahl method; the total sulfur gravimetrically as sulfate after oxidizing the organic matter; the tyrosine and tryptophan by the method of Folin and Marenzi (9). Table I shows the analytical results.

ELECTROPHORESIS STUDIES

Through the kind cooperation of Drs. D. A. MacInnes and L. G. Longworth of Rockefeller Institute we were able to obtain an electrophoretic analysis of the water soluble fractions. All of the patterns were obtained with approximately 1 per cent solutions of the protein on a dry weight basis—in a buffer 0.025 molar in both Na_2HPO_4 and NaH_2PO_4 after electrolysis for 6000–6500 seconds at 7.3 volts per cm.

Included for comparisons are (a) a pattern of undried crystallized ovalbumin (supplied by Dr. MacInnes), (b) a pattern of our dried crystallized ovalbumin (Preparation I), (c) a pattern of heat denatured but not coagulated ovalbumin (Dr. MacInnes). Dr. MacInnes has carried out an electrophoretic analysis of a sample of dried ovalbumin submitted to him by Dr. A. G. Cole. The pattern (b) of our sample of dried ovalbumin corroborates the findings of Dr. MacInnes on Dr. Cole's sample that the dried ovalbumin is less homogeneous electrophoretically than the undried protein (Fig. 1).

The coagulated ovalbumin used in this experiment was completely insoluble in water and in dilute acids (pH 3.0), and alkali (pH 9.5). The ground powder has a light yellow color and a peculiar characteristic odor. The water soluble fractions produced by grinding are not coagulable by heat, are soluble in acids, in alkali and give a Biuret test (pink-violet) indicative of the lower protein degradation products. They are precipitated by half saturation with ammonium sulfate, trichloroacetic acid, phosphotungstic acid, and picric acid.

Preparations II and III contain high concentrations of dialyzable material, 89.77 per cent and 85.5 per cent respectively of the total nitrogen passing through Visking casing membranes upon complete dialysis.

After extraction of the water soluble protein from the ground coagulated ovalbumin, the residue contains fractions which are soluble at pH 3.0 (HCl) and pH 9.5 (NaOH).

As has been pointed out, patterns (a), (b), and (c) have been included for comparison. The water soluble protein from the second 48 hour period of grinding (Preparation III) dissolved or dispersed in the buffer solvent with very little material remaining in suspension that could be

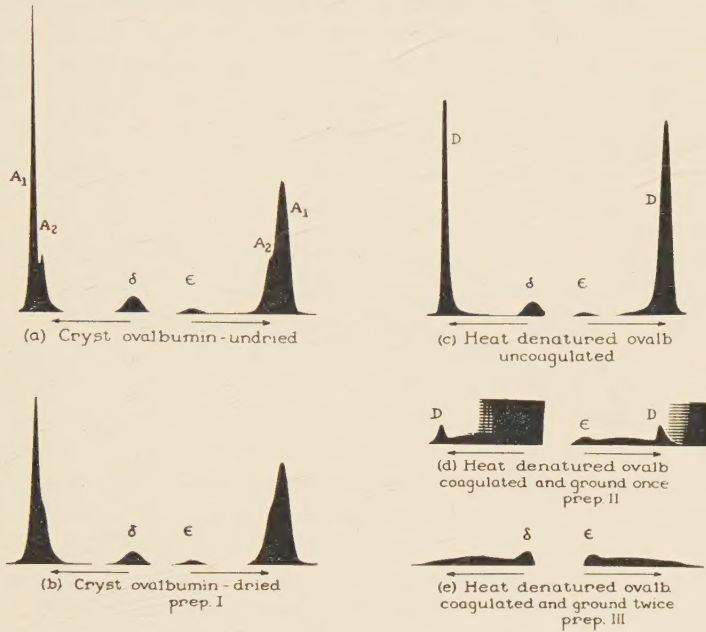


FIG. 1

Electrophoretic Analysis of Dried, Undried, Heat Denatured Ovalbumin and the Water Soluble Fractions Obtained by Grinding Heat Coagulated Ovalbumin

thrown out in the angle centrifuge, but the resulting solution had a pronounced opalescence. In distilled water the solutions are perfectly clear. The pattern (e) of this solution indicates fractions having all mobilities from zero to that of native ovalbumin. No one fraction appears to predominate. This behavior is in marked contrast to that of heat denatured but uncoagulated ovalbumin (c) in which the denatured protein is quite homogeneous electrically and has a mobility very similar to that of the native protein. A striking feature of the pattern

of the ground water soluble protein, however, is the relative absence of refracting material. Only about 45 per cent of the original material is accounted for by the total area under the refractive index gradient curve. This is due to loss of protein in the dialysis prior to electrophoresis, the grinding process having degraded the coagulated protein to a considerable extent.

The pattern (d), which represents Preparation II and is the water soluble protein split off during the first 48 hour period of grinding, shows some material (d) with the original mobility, the effect of grinding not having gone so far. It would appear that one fraction of the water

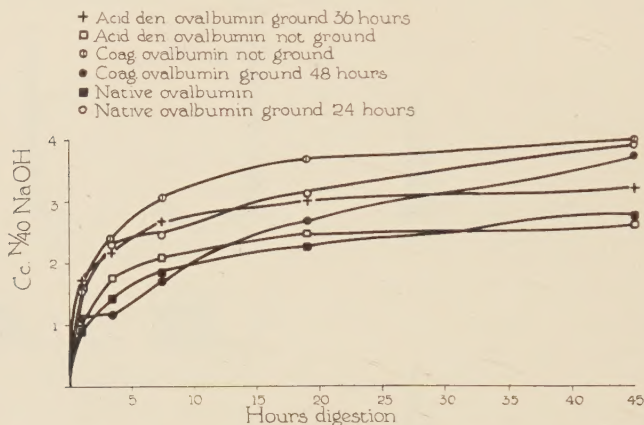


FIG. 2

Comparative Digestion Rates of Various Ovalbumins by Pepsin. Rates Determined by the Sorensen Titration Using $N/40$ NaOH

soluble protein of Preparation II has the mobility of the denatured ovalbumin.

Comparison of Rates of Digestion of Ground and Unground Ovalbumin: The digestion was carried out using Parke, Davis and Company pepsin (1-10,000). The concentration of protein in each digest was exactly the same and equal to 25 mg. of protein either dissolved or suspended in each cc. The suspensions were shaken at frequent intervals. The concentration of enzyme was 4.6 mg. of pepsin protein in each cc. The pH was 1.7 and the digestion was carried out at 37°C.. The digestion was followed by the Sorensen titration (Fig. 2).

From the curves it is readily seen that the unground coagulated oval-

bumin is digested at the most rapid rate. The ground coagulated ovalbumin, from which the water soluble protein has been extracted is digested at a much slower rate indicating differences in the two insoluble proteins. The water insoluble protein produced by grinding native ovalbumin for 24 hours is digested at a slower rate than the heat coagulated egg albumin which has been ground for two 48 hour periods and extracted free of water soluble protein. The ground insoluble acid denatured ovalbumin is digested at a slower rate than heat coagulated ovalbumin but is digested at almost the same rate as the ground insoluble native ovalbumin during the first 20 hours. The acid denatured ovalbumin which has been ground for 36 hours and is insoluble at pH 3.0 is digested more rapidly than the unground acid denatured ovalbumin.

DISCUSSION

It has been mentioned that the coagulated ovalbumin after grinding acquires a definite yellow coloration and a peculiar characteristic odor. This is true of all the proteins studied up to this time and may be due to decomposition of the aromatic amino acids.

The grinding of native ovalbumin brought about definite irreversible changes in the protein molecule. This change is evidenced by the conversion of the water soluble protein into one which is insoluble in water. Since some of the ground water insoluble protein is soluble in acid (pH 3.0) and alkali (pH 9.5) and the major portion is insoluble, it would seem that the conversion occurs in at least two stages. The first is a transformation of the water soluble protein into a protein soluble in acids and alkali, not unlike a denatured protein. The second stage is the transformation into a highly insoluble protein resembling coagulated ovalbumin in its solubilities.

The grinding of acid denatured ovalbumin brings about definite changes in the molecule. After grinding the powder contains little protein soluble in acid and alkali. This might possibly be explained on the assumption that the changes are merely intramolecular in character, involving the weaker secondary valence bonds.

The dry grinding of coagulated ovalbumin breaks down this insoluble protein so that water soluble protein results. The water soluble protein obtained after grinding heat coagulated ovalbumin contains a higher percentage of sulfur, a slightly lower nitrogen content and a lower tryptophan and tyrosine content than the native ovalbumin. The water

soluble fraction, split off during the first 48 hours of grinding, contains no tryptophan.

The electrophoretic analysis indicates that there may be a stepwise degradation of the protein produced by the grinding process. It would appear from the results that the effect of dry grinding has been to produce a most decided disintegration of the protein material.

SUMMARY

1. The dry grinding of native crystallized ovalbumin yields a highly insoluble protein somewhat resembling coagulated ovalbumin.

2. Grinding acid denatured ovalbumin produces an acid (pH 3.0) insoluble protein.

3. The grinding of heat coagulated ovalbumin produces water soluble protein.

4. The sulfur, nitrogen, tryptophan and tyrosine content of these water soluble fractions has been determined.

5. Electrophoretic analysis of the water soluble fractions from heat coagulated ovalbumin indicates that there may be a stepwise degradation of the protein produced by the grinding process.

6. The results indicate a decided disintegration of the protein molecule.

7. The digestion rates of native, ground native, coagulated, ground coagulated, denatured and ground denatured ovalbumin have been compared.

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The Effect of Dry Grinding on the Properties of Proteins*

2. Studies on Casein

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INTRODUCTION

In a previous communication (1) the effect of dry grinding on native ovalbumin, denatured ovalbumin, and coagulated ovalbumin was reported. Soluble proteins are converted into a less soluble form, and insoluble proteins are converted into water-soluble fragments. It is evident that dry grinding produces definite changes in the protein molecule. The effect of dry grinding on casein is presented.

EXPERIMENTAL

25 grams of casein (Code 1559, General Chemical Co., N. Y.) the purity of which was undetermined, were washed completely free of water-soluble protein and placed in a ball mill of 8 liters capacity and ground for 48 hour periods. Four water-soluble fractions were obtained. Upon these four water-soluble proteins and the water-insoluble residue remaining after four successive periods of grinding, the action of rennin and the phosphorus, nitrogen and tryptophan content were determined. The action of rennin was carried out on the fractions dissolved in a buffer of pH 6.5. Phosphorus was determined by the Neumann-Pemberton (2) method and nitrogen by the Kjeldahl method. The May and Rose (3) method was used to determine tryptophan (Table I).

RESULTS

The water-soluble protein obtained by the action of grinding during the first 48 hour period had a higher phosphorus content and a lower

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percentage of tryptophan than that of unground casein; rennin acted on this fraction yielding a loose clot. The other fractions were not affected by rennin but contained more phosphorus than native casein. All the water-soluble fractions were low in tryptophan. The low tryptophan content of these fractions may be taken to indicate that the grinding process breaks specific linkages in the casein molecule, splitting off water-soluble fractions having a low concentration of this amino acid or that tryptophan is destroyed by the grinding process. There is little difference in the percentage of nitrogen in the different fractions.

The first water-soluble fraction is precipitated by acetic acid. On cooling the hot solution, the protein reprecipitates, a maximum amount

TABLE I
Chemical Differences in the Water-Soluble Fractions, Native Casein, and Residue

Sample	Rennin Action	Phos- phorus <i>per cent</i>	Nitrogen <i>per cent</i>	Trypto- phan <i>per cent</i>	Water-soluble Protein from 25 g. Casein <i>g.</i>
Washed Casein.....	+	0.85	15.70	2.2	25
1st.....	+	1.15	15.28	0.44	5.5
2nd.....	—	1.19	15.61	0.47	3
3rd.....	—	1.26	15.61	0.64	2
4th.....	—	1.05	15.04	0.69	2.5
H ₂ O Insol. residue after 4 grindings.....	+	0.67	15.35	1.38	16.1

forming at 50–60°C. When the solution has reached room temperature most of the protein has redissolved.

The second water-soluble fraction is not precipitated by acetic acid but in water solution gives the same type of reaction as described for the first fraction. In acid solutions (pH 4.5) a higher temperature is required to bring about coagulation than that necessary when the protein is dissolved in distilled water. There is also a lesser quantity of coagulum formed in solutions of this acidity.

The third and fourth water-soluble fractions are not precipitated by acid and do not coagulate in solutions of acidity below pH 4. These fractions dissolved in distilled water are coagulable.

The fractions all contain dialyzable proteins. The water-soluble proteins are precipitated from solutions in water by trichloroacetic, picric acid, mercuric chloride, phosphotungstic acid, and half saturated am-

monium sulfate. Although the first fraction is precipitated by acetic acid, the other fractions are not precipitated by acids or alkali. All water-soluble proteins give a pink-violet biuret test, indicating a smaller molecular size.

The water-soluble proteins are not precipitinogenic but produce anaphylactic sensitization in guinea pigs.

Comparison of Tryptic Digestion Rates of the Casein Fractions

Solutions of the proteins were made up in a phosphate buffer at pH 8.5. The concentration of protein in all of the digests was the same and equal to 31.6 mg./cc. The enzyme used was trypsin (Fairchild) and the

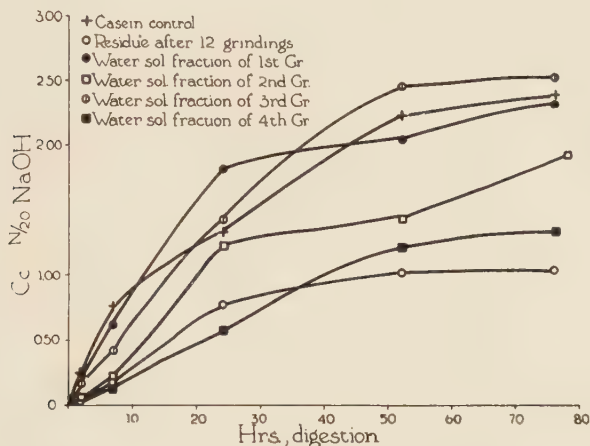


FIG. 1

Comparison of Rates of Digestion of Water-Soluble Proteins of Casein and Native Casein by Trypsin

amount added was such as to give a concentration of 3.16 mg./cc. 3 cc. of 5 per cent thymol in alcohol for 50 cc. of digest was used as a preservative. Digestion was carried out at 37° C. The rate of hydrolysis was followed by the Sørensen titration using N/20 NaOH (Fig. 1).

The results indicate that prolonged grinding with removal of the water-soluble protein formed leaves a residue which is only slowly digested by trypsin. The water-soluble proteins are all digested much more readily than the water-insoluble residue, although the water-soluble protein of the fourth grinding is only slowly attacked by the enzyme during the first 34 hours of hydrolysis. The water-soluble fraction of the first grinding is

hydrolyzed at a faster rate during the first 45 hours than is native casein. The hydrolysis of this fraction has almost reached its maximum at the end of this period. In the case of the native protein maximum hydrolysis requires at least 50 hours.

It is quite evident from these studies that the water-soluble proteins produced by grinding are different from one another, although their content in nitrogen, phosphorus, and tryptophan, and the effect of heat on their solutions indicate some degree of similarity.

Water-Soluble Casein in Nutrition

500 grams of dry Harris vitamin free casein, extracted free of water-soluble protein, were ground in a ball mill of 8 liters capacity for successive periods of 48 hours each. After each 48 hours the ground powder was removed from the mill and extracted with distilled water until free of the water-soluble protein produced by the grinding. The dried insoluble

TABLE II
Analysis of Proteins Used in Nutritional Studies

Fraction	Phosphorus <i>per cent</i>	Sulfur <i>per cent</i>	Tryptophan <i>per cent</i>
Water-Soluble.....	1.27	0.45	0.53
Water-Insoluble.....	0.68	0.089	1.86
Original Casein.....	0.85	0.78	2.20

residue was again subjected to further grinding. By 12 successive grindings 40 grams of water-soluble protein were obtained. This was utilized for the study of the nutritional value of the water-soluble component as compared with that of unground casein and the water-insoluble residue remaining after the grinding process. The phosphorus, sulfur, and tryptophan content of these fractions have been determined (Table II).

It was desirable to determine the adequacy for growth of the water-soluble protein produced from casein since it gives some indication as to its content in the essential amino acids.

White mice were used in these experiments because of the limited quantity of the water-soluble casein available for use in making up the diet. The Sherman-Sandels diet was used and contained casein 18 g., cornstarch 52 g., salt mixture 4 g., butterfat 7 g., autoclaved yeast 15 g., and cod liver oil, 3 g.

Three mice were fed on a diet with the combined water-soluble protein obtained by successive grinding extraction and grinding of Harris casein.

Another three mice were given a diet with residue remaining after 12 successive grindings as the protein in the diet. A third series of three mice were given a diet with unground casein which had been completely extracted free of water-soluble proteins. The growth curves for the diets are given in Fig. 2. The phosphorus, sulfur, and tryptophan of the water-soluble protein and the water-insoluble residue after 12 grindings were determined by methods described previously (Table II).

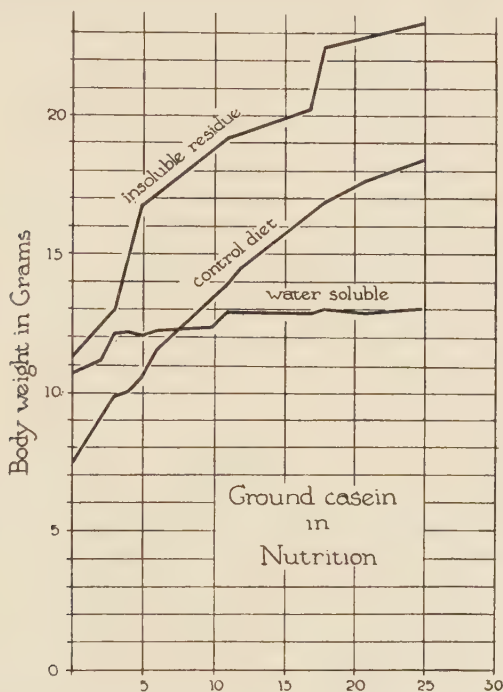


FIG. 2

The Value of Water-Soluble Casein in Nutrition of Mice Compared With That of the Insoluble Residue and Native Casein

The results definitely show that the water-soluble casein is nutritionally deficient since it does not support growth in mice. The insoluble residue was just as effective in maintaining the animal in good health and for growth as the native casein. It would thus appear that prolonged grinding does not affect the essential amino acids greatly since a complete lack of any of these in the ground residue would have produced a deficient diet.

Totter and Berg (4) have shown that mice will grow on a diet previously deficient in tryptophan if the natural amino acid is added to the diet in a concentration of 0.1%. The addition of 0.2% of the *l*-amino acid tryptophan to the deficient diet produced much better growth. Fig. 2 indicates that the water-soluble protein of ground casein, although capable of maintaining the animal in good health and at a relatively constant weight, was at least partially deficient from a nutritional standpoint. Since the tryptophan content of the diet containing the water-soluble protein was approximately 0.1%, the lack of growth was due to a partial deficiency of some other amino acid or acids, other than tryptophan in the diet. Because of the small number of animals used in this experiment, further studies will have to be carried out before definite conclusions can be made.

How Does Grinding Produce Degradation of the Protein Molecule?

It has been shown that water-soluble proteins are converted by dry grinding into an insoluble form. The dry grinding of proteins insoluble in water produces water-soluble fragments. From the available data the conclusion seems justifiable that definite linkages in the protein molecule are attacked.

The water-soluble proteins, if we assume a Wrinch structure, would become insoluble through an unfolding of the cage molecule, similar to that which occurs as a result of surface spreading.

The transformation of the soluble protein into the insoluble form is apparently only a preliminary step in the alteration of the protein molecule by the action of dry grinding. The linkages broken at this point must be the weak secondary valence bonds, thus producing intramolecular changes. The grinding of these insoluble proteins and those insoluble in the native state produce further changes which lead to the splitting off of water-soluble fragments. The linkages involved here are not known. In studies on gelatin (see page 353), it will be shown that detectable peptide splitting did not occur. The possibility exists that while wholesale peptide splitting did not take place, such bonds could have been broken but in numbers too few to be determined.

That the soluble protein is produced by the action of microorganisms acting during extraction of the dry ground powder is unlikely since these soluble fractions are immediately extractable by distilled water.

It would be interesting to know by what mechanism this fragmentation of the protein molecule is brought about. Routh (5) has discussed

the rôle played by the composition of the mill and balls on the grinding process. The temperature does not increase since the mill and its contents are only slightly warmer than the external atmosphere. However, at the point of contact of the balls a large amount of energy as heat may be liberated and this may be one of the factors influencing the changes produced. The odor which is observed with all proteins after they have been ground may be associated with this heat effect. The balls as they strike each other may constitute a shearing force which 'slices' the protein into at least two fragments, one of which is at least soluble in water.

CONCLUSIONS

1. Water-soluble proteins are produced when casein is dry ground.
2. The water-soluble proteins contain a higher content of phosphorus than native casein.
3. The fractions all have a low tryptophan content.
4. The first water-soluble protein fraction was acted upon by rennin but the others were not.
5. The water-soluble protein is partially deficient but maintains mice in constant weight.

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The Effect of Dry Grinding on the Properties of Proteins*

3. Gelatin

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INTRODUCTION

Alsberg and Griffith (1) state that dry grinding renders gelatin cold-water-soluble. Solutions of ground gelatin in distilled water form gels after standing for some time. Alsberg considers the changes observed to be of a purely physical nature. Boissevain (2) repeated these studies and found that gelatin ground in a ball mill for 96 hours became perfectly water-soluble. A 10 per cent solution of this gelatin would not gel even after standing in the ice box for a week. Boissevain explains the changes by assuming that hydrolysis of the gelatin into peptone had occurred. Short periods of grinding apparently had no effect on the gelatin properties of this protein.

EXPERIMENTAL

Fifty grams of Knox granular gelatin were placed in a ball mill of 7 liters capacity and the mill rotated at about 100 R.P.M. Samples were removed at intervals of time, the total time of grinding being 72 hours.

Solutions of definite concentrations of the ground samples were prepared by dissolving the necessary quantity of the dried protein in distilled water at room temperature. Unground gelatin was dissolved at 60°C. and allowed to cool to room temperature. The solutions were placed in a water bath at 5°C. and the time required for gel formation was determined. The results are tabulated in Table I.

To determine whether the change in solubility was due to a hydrolytic

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process, as suggested by Boissevain, the Sørensen titration was carried out upon 1 gram samples of protein dissolved in distilled water using 0.05 *N* NaOH (Table II).

It is generally known that gelatin heated about 70°C. for a number of hours loses its ability to gel. Unground gelatin which in 2 per cent concentration formed a gel in 3 minutes required 31 minutes to gel after heating for 18 hours at 80°C. Gelatin ground for 7 hours and largely

TABLE I
Effect of Grinding Gelatin on Time for Gel Formation

Hours Grinding	Time for Gel Formation Concentration of Protein			
	2%	3%	4%	5%
0	3 min.			
7	3 min.			
22	12 min.			
30	14 min.			
48	24 hrs.	6 hrs.	1 hr.	17 min.
54	*	*	*	15 hrs.
72	*	*	*	*

* No gel formed even after 10 days at 5°C.

TABLE II
Formal Titrations of Ground Gelatin

Hours Grinding	cc. 0.05 <i>N</i> NaOH per g.
0	5.04
7	5.05
22	5.07
30	4.98
48	5.00
54	5.03
72	4.95

soluble in cold water and which also formed a gel in 3 minutes required standing for 2 hours before a gel formed after being similarly treated.

RESULTS

It is evident that the longer the period of grinding the more drastic is the effect on the protein molecule. The grinding of gelatin for 7 hours has no effect on its ability to form a gel but there is a marked increase in solubility in water at room temperature. That there are other differences is indicated from the results obtained by heating the ground

and unground samples of gelatin, the former requiring 4 times as long for gel formation as the latter.

The formal titration values indicate that there was no appreciable splitting of peptide bonds. Boissevain explained his results by assuming a hydrolytic process with conversion of the gelatin into peptone. The change in the physical properties of the gelatin could be explained by the assumption that peptide bonds may have been broken. The splitting of secondary valence bonds such as hydrogen bonds and disulfide bonds of cystine might produce similar results.

DISCUSSION

The ability of gelatin solutions to form gels is according to Katz and Gerngross (3) dependent on the arrangement of the molecules into a definite structure. Since short periods of grinding have little effect on gelatin, other than to increase its solubility in cold water, it would seem that the early changes produced are not drastic but merely serve to open up linkages leading to a greater number of polar groups in the molecule and thus increased solubility. Prolonged grinding produces more deep seated changes.

The increased solubility cannot be explained on the basis of a mechanical process bringing about a greater availability of the material to solvent action. Nor is it possible to consider the differences in ground and unground gelatin as due to hydrolytic action alone as Boissevain believes.

SUMMARY

1. The effect of grinding gelatin is to convert it into a cold water-soluble protein.
2. The gel formation time increases with increase in the time of grinding.
3. There is no increase in the formal titration value during grinding.

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The Effect of Dry Grinding on the Properties of Proteins*

4. Human, Beef, and Hog Coagulated Hemoglobins

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INTRODUCTION

In previous papers (1) we have described the effect of dry grinding on various forms of ovalbumin, gelatin, and casein. In this paper we report the effect of dry grinding on human, beef, and hog coagulated hemoglobins.

EXPERIMENTAL

The method of Marshall and Welker (2) was used for the preparation of the hemoglobin. The clear dilute (0.2 per cent) solutions of hemoglobin were adjusted to pH 6.8 with molar K_2HPO_4 and the solutions placed in a boiling water bath for 30 minutes. The dark brown coagula were filtered off, washed with boiling water, and spread upon glass plates to dry.

The dry proteins were separately ground in a ball mill of 8 liters capacity for successive periods of 48 hours each. After each 48 hour period, the water-soluble protein was extracted from the powder with distilled water. The water-soluble protein was obtained in dry form by passing dry warm air at 38°C. over the solutions. The insoluble residues were similarly dried before being subjected to further grinding.

From coagulated human hemoglobin we prepared 8 water-soluble fractions, 4 from coagulated beef hemoglobin and 2 from coagulated hog hemoglobin.

The percentages of nitrogen, iron, tyrosine, and tryptophan of these fractions were determined. Nitrogen was determined by the Kjeldahl

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method, iron by MacFarlane's method (3) (the Pulfrich Photometer was used instead of the ordinary colorimeter), tyrosine and tryptophan by the Folin and Marenzi method (4). The amount of dialyzable nitrogen was determined by exhaustively dialyzing solutions of known concentration through Visking dialysis shells (Table I).

TABLE I
Water-Soluble Fractions of Coagulated Ground Hemoglobin

Sample	N ₂	Fe	Tyrosine	Tryptophan	Amount of Water-Sol. Extract by H ₂ O	Diffusible Nitrogen
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent of orig.</i>	<i>per cent</i>
<i>Human Hemogl.</i>						
1st.....	15.54	0.2505	2.47	0.41	16.6	77.95
2nd.....	15.30	0.2709	2.12	0.72	25	95.09
3rd.....	15.35	0.276	1.98	0.76	8.7	84.53
4th.....	15.21	0.420	2.09	0	6	93.36
5th.....	14.90	0.420	2.02	0	5	88.12
6th.....	14.52	0.370	1.86	0	8.3	69.93
7th.....	12.79	0.390	1.62	0	5.4	83.79
8th.....	11.72	0.362	1.61	0	5	78.25
<i>Ox Hemogl.</i>						
1st.....	15.11	0.174	2.74	0.61	21.5	
2nd.....	15.20	0.370	2.42	0.72	32.5	
3rd.....	14.65	0.354	2.45	0	10	
4th.....	13.25	0.285	2.75	0.75	10	
<i>Hog Hemogl.</i>						
1st.....	15.55	0.228	1.85	0.72	17.5	
2nd.....	15.31	0.336	2.42	0.54	15	

RESULTS

As seen in Table I the water-soluble fractions contain iron in variable amounts. They all give the benzidine reaction. The fact that the hematin is soluble in water indicates that the prosthetic group is not split from the protein moiety. The manner in which the hematin is attached to the protein remains unknown but it is quite definite that this linkage is not split by the grinding procedure.

The water-soluble proteins contain dialyzable protein, are non-coagulable by heat and require concentrations of 50% or more of alcohol for precipitation. They are precipitated by mercuric chloride, picric

acid, trichloroacetic acid, and by half saturation with ammonium sulfate. They are soluble in acids and alkali. The indications are that the fragments produced by grinding are small in molecule size, probably that of primary and secondary proteoses. The water-soluble fractions do not give rise to precipitin antibodies and do not react with native hemoglobin antisera.

The nitrogen content decreases with successive fractions which may be interpreted as a loss of ammonia by deamination during the grinding process. This seems also to be true for the tyrosine in human hemoglobin while that of the soluble fractions of beef hemoglobin is fairly constant.

The distribution of tryptophan in the fractions is also quite interesting. This amino acid is absent in the last four fractions of human hemoglobin and in one of the water-soluble fractions of the beef hemoglobin.

79% of the coagulated human hemoglobin was converted into water-soluble protein in 384 hours grinding. If the grinding had been continued all of the coagulated hemoglobin might possibly have been obtained as soluble protein. 75% of the coagulated beef hemoglobin was converted in 192 hours grinding, while 32.5% of the coagulated hog hemoglobin became soluble in 96 hours.

The water-soluble fractions from human hemoglobin contain at least 70% dialyzable nitrogen, thus indicating that they are small molecular fragments. The hematin remained unaffected by 300 hours of continuous grinding since it was possible to recover from the ground powder the theoretical yield of hematin by recrystallization from a chloroform and quinine solution.

Spectrographic Analysis

The analysis was carried out using a Hilger Quartz Spectrograph, spectra being photographed upon Eastman Orthochromatic plates. Ultra violet spectra were obtained by the use of a Kromayer mercury arc lamp. All plates contain one comparison spectrum transmitted through an empty cell and a series of spectra through the same cell filled with the material being studied. The time of exposure was 50 seconds, the concentration of protein was 0.05 per cent. The light portion of the plates are areas affected by transmitted light. A normal spectrum of the native hemoglobin and of hematin (.002 per cent) dissolved with the aid of alkali is included for comparison. A portion of the visible spectrum is faintly seen at about 540 and 570 μ (Figs. 1, 2, 3).

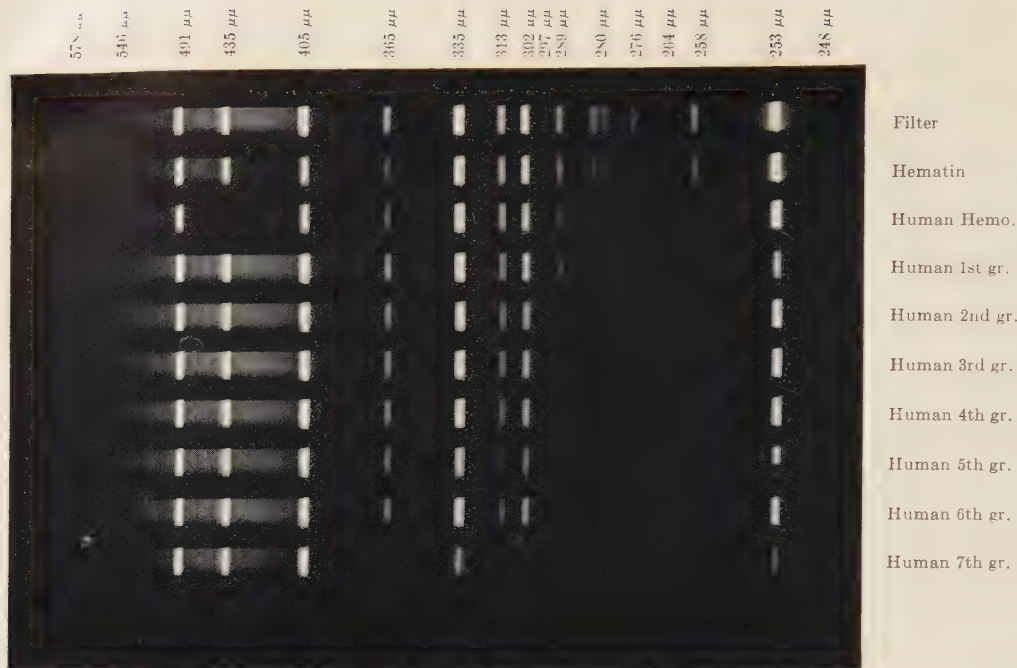


FIG. 1 Spectra of Soluble Fractions of Coagulated Human Hemoglobin

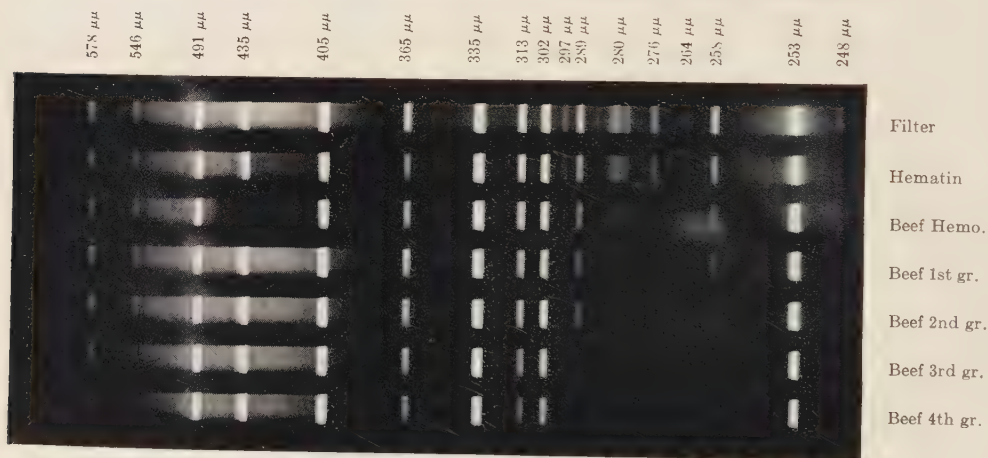


FIG. 2 Spectra of Soluble Fractions of Coagulated Beef Hemoglobin

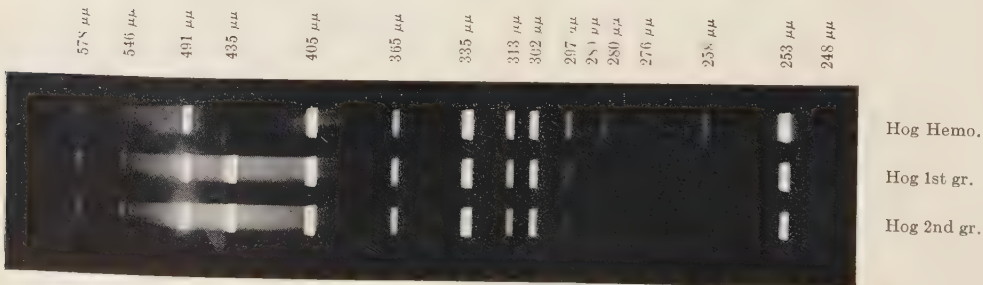


FIG. 3 Spectra of Soluble Fractions of Coagulated Hog Hemoglobin

RESULTS

The water-soluble fractions seem to differ from native hemoglobin mainly in the region of the ultraviolet from 313 $\mu\mu$ to 264 $\mu\mu$. At 313 $\mu\mu$ they absorb more ultraviolet light than does the native protein. The water-soluble fractions of coagulated human hemoglobin absorb more strongly at 297 $\mu\mu$ as we go from the 1st to the 8th grinding. This is also true at 280, 276, and 264 $\mu\mu$. At 258 $\mu\mu$ and 248 $\mu\mu$ absorption by the ground fraction is complete in all cases except that of the first fraction, less than 1% being transmitted at 248 $\mu\mu$.

The similarity in the ultraviolet absorption of all three hemoglobin fractions, suggests the possibility of the existence of similarity in the structure of the molecules of hemoglobins studied. The protein fragments split off by the grinding process may possibly have a similar structural arrangement.

DISCUSSION

It is apparent that the grinding process brings about changes in the three coagulated hemoglobins similar to those observed in our study of coagulated ovalbumin. As in the case of the latter protein a stepwise degradation of the protein molecule is effected. The high quantity of diffusible nitrogen and the precipitability of the protein in the water-soluble fractions by various reagents indicates that small molecular weight particles are present. Data from the Laboratory of Dr. Hans Neurath, received after the paper on ovalbumin was reported, on diffusion measurements of the water-soluble fractions of coagulated ovalbumin indicates that the particles of lowest molecular weight are 10,000--12,000.

CONCLUSIONS

1. Water-soluble protein is produced when coagulated hemoglobin is dry ground.
2. The nitrogen and tyrosine content of the water-soluble proteins decreases with successive grinding.
3. There occurs during grinding a most decided degradation of the protein molecule.

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The Effect of Dry Grinding on the Properties of Proteins*

5. Keratins

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A. WOOL

Native keratins are attacked by the enzymes at a very slow rate. Michaelis and Goddard (1) have shown that wool becomes more soluble and digestible if first treated with certain reducing agents. The reduction occurs at the disulfide bond of cystine, splitting them, and producing smaller fragments.

Routh and Lewis (2) compared the rates of digestion of keratin and ground wool. They found that wool reduced by thioglycolic acid (kerateine) was digested by both trypsin and pepsin at a greater rate than the ground wool. The ground wool was shown to contain appreciable amounts of water-soluble protein, a portion of which was dialyzable. These authors explained the presence of water-soluble protein in the powdered wool "by mechanical action which made the soluble material accessible to the solvent." This view alone cannot explain the observed effects on the keratins, reported here, and the other proteins studied as previously reported (3, 4, 5, 6).

EXPERIMENTAL

Defatted sheep wool was ground in an 8 liter ball mill for 72 hours. The wool ground for 72 hours was partly soluble in water, only 12% of the total protein dissolving. 11% of the ground wool dissolved in 0.12 *N* HCl and 21.3% dissolved at pH 8.6.

Thioglycolic acid wool was prepared by the method described by Michaelis and Goddard.

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Tryptophan determinations by the May and Rose (7) method showed that none of the fractions contained this amino acid.

A comparison of the rates of digestion by trypsin and pepsin of the ground and thioglycolic acid reduced wool has been carried out. For the pepsin experiment the proteins were suspended in a 0.12 N HCl and for the tryptic digests, a boric acid buffer at pH 8.6 was used. The concentration of protein in each case was 20 mg./cc. The rate of digestion was determined by the increase in soluble nitrogen determined by the Kjeldahl method (Fig. 1).

Results

Keratin, like other water-insoluble proteins, yields water-soluble fractions after being dry ground in the ball mill. The water-soluble proteins have a characteristic odor which is acquired by all proteins after grinding. As neither the water-soluble fractions nor the water-insoluble residue remaining after grinding contained tryptophan, it would appear that this amino acid was destroyed by the grinding process.

The findings of Routh and Lewis have been corroborated. Fig. 1 shows that kerateine is digested more rapidly than ground wool by both trypsin and pepsin.

The ground wool gives a direct nitroprusside test indicating the splitting of disulfide bonds. This test is given by all of the sulfur-containing proteins thus far ground. However, the importance of the breaking of this linkage in producing water-soluble protein cannot be too great. The fact that ground wool is digested at a slower rate than the reduced wool would point to the possibility that other linkages were being broken in grinding. Other linkages, such as hydrogen bonds and a few peptide linkages, if broken would result in fragments being produced having greater solubility. Polypeptide chains may be split from the polypeptide grid. Since proteins having a low sulfur content (gelatin, casein, elastin) also yield water-soluble proteins, thus undergoing fragmentation, it would seem that the disulfide bond is only partially responsible in the high sulfur containing proteins and only of minor importance in the low sulfur proteins for the breakdown observed.

B. OX-HORN KERATIN

20 grams of ox-horn keratin were obtained by shaving the horn of an ox and placed in a large ball mill and ground for 48 hours. After this time the powder was extracted and the grinding repeated until four such fractions, soluble in water, were obtained. These fractions have been analyzed for their nitrogen, sulfur, tyrosine, and tryptophan content. The amount of nitrogen diffusible through Visking casing shells was determined (Table I).

To determine the digestibility of the water-soluble proteins by pepsin and trypsin, the fractions were dissolved so that the protein concentration was 20 mg./cc. The solutions for peptic digestion were made up in 0.63% HCl and for

TABLE I
Analysis of the Water-Soluble Proteins of Ox Horn Keratin

Protein	Nitrogen per cent	Sulfur per cent	Tyrosine per cent	Trypto- phan per cent	Dialyz- able per cent
Native Keratin*.....	15.73		4.80	1.8	
Keratin 1st gr.....	15.32	4.16	3.41	0	74.3
" 2nd gr.....	15.13	4.32	3.54	0	76.4
" 3rd gr.....	14.97	4.02	3.67	0	80.3
" 4th gr.....	14.51	4.36	4.14	0	72.7
" Residue*....	13.02		3.31	0.67	

* Values corrected for the ash content.

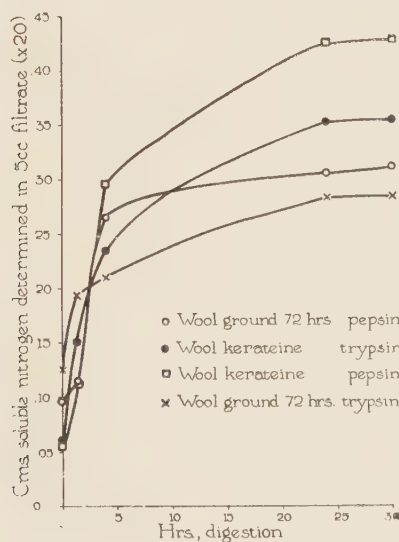


FIG. 1

Comparison of the Digestion of Ground Wool and Wool Keratin by
Trypsin and Pepsin

the tryptic experiment in a buffer at pH 8.2. The rate of hydrolysis was followed by the Sorensen titration. The pepsin concentration was 10 mg. in each cc. (1-3,000 pepsin). The trypsin (Fairchild) was added so that the ratio of substrate to enzyme was 4-1 (Figs. 2 and 3).

Results

As shown with some of the other proteins studied, the nitrogen content decreases in successive water-soluble fractions. They contain no tryptophan, since no color developed when the May and Rose method was used. Tyrosine is fairly constant in the first three fractions, that of the fourth being somewhat higher. All fractions contain over 4% sulfur although the state of the sulfur, whether organic, inorganic or both, remains undetermined.

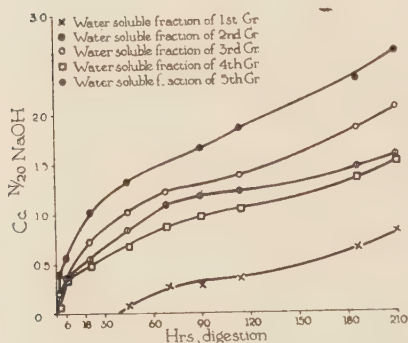


FIG. 2

FIG. 2.—Comparison of Rates of Digestion of Water-Soluble Proteins from Ox Horn Keratin by Pepsin.

Formal titration using $N/20$ H_2SO_4 .

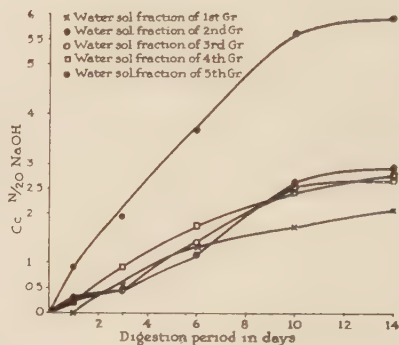


FIG. 3

FIG. 3.—Comparison of Rates of Digestion of Water-Soluble Proteins from Ox Horn Keratin by Trypsin.

Formal titrations using $N/20$ NaOH .

A comparison of the rates of digestion by trypsin and pepsin indicates that the second water-soluble fraction is digested at a more rapid rate than the others. According to Routh, the water-soluble protein split from wool is not attacked by trypsin or pepsin. This points to the possibility of there being a distinct difference in the structure of wool and ox-horn keratin.

The absence of tryptophan in the water-soluble proteins and the low concentration of this amino acid in the water-insoluble residue would seem to indicate a marked destruction of tryptophan in the grinding process.

Some protein may be extracted from the ground ox-horn keratin by 95% alcohol. This protein is soluble in alcohol-ether mixture, is precipitated from solution by the addition of water and gives the Biuret reaction.

The water-soluble fractions give a violet biuret test. They are precipitated by phosphotungstic acid, picric acid, by half-saturation with ammonium sulfate, and yield only slight precipitates with trichloroacetic acid. They are non-coagulable by heat and are not precipitated by acid or alkali.

CONCLUSIONS

We have pointed out that the dry grinding of various types of proteins brings about wholesale degradative changes. Similar changes have been shown to take place in wool and ox-horn keratin. The production of water-soluble protein is explained more satisfactorily by the above facts, rather than by a viewpoint held by other workers that the process is a purely physical effect.

The fibrous proteins are believed to have a grid structure consisting of polypeptide chains. The importance of lateral links between parallel polypeptide chains, the bond of the disulfide group of cystine and the polar link of diamino and dicarboxylic acids in determining the stability of keratin has been shown by Speakman (8). The water-soluble proteins are formed perhaps because of the breaking of these bonds as well as others in the grinding process.

Kerateine is formed by reducing agents due to conversion of disulfide to sulfide. Since thioglycolic wool is different from wool which has been ground, it would seem that other linkages were also attacked in dry grinding which are unaffected by reduction. The use of this method may prove a valuable aid in determining structural details in keratin as well as of other proteins.

SUMMARY

1. Ox-horn keratin, like wool, yields water-soluble protein upon grinding.
2. The water-soluble fractions contain no tryptophan.
3. The nitrogen content decreases with successive grindings, a fact noted with some of the other proteins studied.
4. The water-soluble proteins contain high percentages of dialyzable material. This indicates that the fragments are of small molecular size.

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The Effect of Dry Grinding on the Properties of Proteins*

6. Peptone-Proteose

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INTRODUCTION

It has been shown by us (1, 2, 3, 4) that the protein molecule can be broken down into smaller, water-soluble molecular components by dry grinding.

We have reported that ground coagulated ovalbumin yields water-soluble fractions which are heterogeneous as shown by electrophoresis and diffusion data. The latter data indicates that the smallest particles have molecular weights of 10,000–12,000.

The results of the analyses reported here are intended to show that the proteose fraction of proteose-peptone mixture is further split or converted into peptone.

EXPERIMENTAL

Fifty grams of Witte Peptone, which contained 14.72% of the total nitrogen as proteoses determined by precipitation with trichloroacetic acid, were ground in a ball mill in a manner previously described (1). Samples of the ground peptone were removed at intervals of time and the amount of nitrogen precipitable by trichloroacetic acid from solutions of these was determined. The change in this value was taken to indicate the change from proteose to peptone.

Tryptophan, tyrosine, total nitrogen, and ash were also determined. Tryptophan was determined by the May and Rose method (5). The content of tryptophan in the casein control was taken as 2.2%. The tyrosine values were obtained from analysis of the fraction by the Folin and Marenzi method (6). Nitrogen determinations were carried out by the macro-Kjeldahl method (Table I).

All samples were brought to constant weight in an electric oven at 70°C. and then kept in a desiccator over sulfuric acid until analyzed.

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4. Tyrosine is destroyed less readily than tryptophan, very little change occurring during the first 152 hours of grinding in the ball mill.

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